A novel ion-exclusion chromatography–mass spectrometry method to measure concentrations and cycling rates of carbohydrates and amino sugars in freshwaters

Horňák, Karel ; Pernthaler, Jakob

Abstract: The concentrations of free neutral carbohydrates and amino sugars were determined in freshwater samples of distinct matrix complexity, including meso-, eu- and dystrophic lakes and ponds, using high-performance ion-exclusion chromatography (HPIEC) coupled to mass spectrometry (MS). In contrast to other methods, our approach allowed the quantification of free neutral carbohydrates and amino sugars at low nM concentrations without derivatization, de-salting or pre-concentration. New sample preparation procedures were applied prior to injection employing syringe and hollow fiber filtration. Analytes were separated on a strong cation exchange resin under 100% aqueous conditions using 0.1% formic acid as a mobile phase. To minimize background noise in MS, analytes were detected in a multiple reaction monitoring scan mode with double ion filtering. Detection limits of carbohydrates and amino sugars ranged between 0.2 and 2nM at a signal-to-noise ratio >5. Error ranged between 1 and 12% at 0.5-500nM levels. Using a stable isotope dilution approach, both the utilization and recycling of glucose in Lake Zurich was observed. In contrast, N-acetyl-glucosamine was equally rapidly consumed but there was no visible de novo production. The simple and rapid sample preparation makes our protocol suitable for routine analyses of organic compounds in freshwater samples. Application of stable isotope tracers along with accurate measures of carbohydrate and amino sugar concentrations enables novel insights into the compound in situ dynamics.

DOI: https://doi.org/10.1016/j.chroma.2014.09.007

Title: A novel ion-exclusion chromatography-mass spectrometry method to measure concentrations and cycling rates of carbohydrates and amino sugars in freshwaters

Author: Karel Horňák Jakob Pernthaler

PII: S0021-9673(14)01387-9
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2014.09.007
Reference: CHROMA 355796

To appear in: *Journal of Chromatography A*

Received date: 21-5-2014
Revised date: 11-8-2014
Accepted date: 2-9-2014

Please cite this article as: K. Horňák, J. Pernthaler, A novel ion-exclusion chromatography-mass spectrometry method to measure concentrations and cycling rates of carbohydrates and amino sugars in freshwaters, *Journal of Chromatography A* (2014), http://dx.doi.org/10.1016/j.chroma.2014.09.007

A novel ion-exclusion chromatography-mass spectrometry method to measure concentrations and cycling rates of carbohydrates and amino sugars in freshwaters

Karel Horňák*, Jakob Pernthaler

Limnological Station, Institute of Plant Biology, University of Zurich, Seestrasse 187, CH-8802 Kilchberg, Switzerland

*Corresponding author. Tel.: +41 44 634 9236; fax: +41 44 634 9225

E-mail addresses: khornak1@gmail.com (K. Horňák), pernthaler@limnol.uzh.ch (J. Pernthaler).

Keywords:

Carbohydrates; Amino sugars; Ion-exclusion chromatography, Mass spectrometry, Stable isotope tracers; Freshwater

Abstract

The concentrations of free neutral carbohydrates and amino sugars were determined in freshwater samples of distinct matrix complexity, including meso-, eu- and dystrophic lakes and ponds, using high-performance ion-exclusion chromatography (HPIEC) coupled to mass spectrometry (MS). In contrast to other methods, our approach allowed the quantification of free neutral carbohydrates and amino sugars at low nM concentrations without derivatization, de-salting or pre-concentration. New sample preparation procedures were applied prior to injection employing glass fiber and hollow fiber filtration. Analytes were separated on a strong cation exchange resin under 100% aqueous conditions using 0.1% formic acid as a mobile phase. To minimize background noise in MS, analytes were detected in a multiple reaction monitoring scan mode with double ion filtering. Detection limits of carbohydrates and amino sugars ranged between 0.2 – 2 nM at a signal-to-noise ratio >5. Error ranged between 1 - 12 % at 0.5 - 500 nM levels. Using a stable isotope dilution approach, both, the utilization and recycling of glucose in Lake Zurich was observed. In contrast, N-acetyl-glucosamine was equally rapidly consumed but there was no visible *de novo* production. The simple and rapid sample preparation makes our protocol suitable for routine analyses of organic compounds in freshwater samples. Application of stable isotope tracers along with accurate measures of carbohydrate and amino sugar concentrations enables novel insights into the compound *in situ* dynamics.

1. Introduction

Dissolved organic matter (DOM) in freshwater ecosystems consists of a wide array of compounds that are in parts linked to processes mediated by aquatic microbes [e.g., 1]. DOM is a key nutrient and energy source for consumers, particularly for heterotrophic bacteria [e.g., 2,3]. Major sources of DOM in freshwaters are autochthonous primary production and the allochthonous input from the terrestrial environment [4-6].

Carbohydrates and amino sugars often constitute a prominent fraction of the labile DOM fraction in aquatic systems [e.g., 7,8]. Yet the quantification of dissolved carbohydrates and amino sugars is analytically challenging due to their typically low *in situ* concentrations at nM levels [9], the chirality at many carbon atoms and the existence of anomers resulting in a large number of isomers. Diverse colorimetric and chromatographic approaches have been used for the determination of environmental carbohydrates. However, the application of colorimetric methods [10-12] has been limited by sensitivity, poor detection limits (~µM) and by time-consuming derivatization steps. Similarly, chromatographic methods employing borate complexes [13,14], reversed-phase chromatography with DNS and p-AMBA [15] derivatives or gas chromatography with ester or acetyl derivatives [16,17] all require difficult sample preparation and their detection limits range between 100 nM to 20 µM.

To date, only high-performance anion-exchange chromatography with pulsed amperometric detection [HPAEC-PAD, 18] allows the detection of carbohydrates and amino sugars with sufficient sensitivity (2-10 nM) and without laborious derivatization procedures. The HPAEC-PAD method has been employed for the quantification of neutral sugars [18], amino sugars [19], various disaccharides [20] or a combination of those compounds [21] in samples from various aquatic habitats. Despite being widely applied, the sensitivity and specificity of the HPAEC-PAD method may be compromised by measurement interferences

with salt anions and a complex sample matrix [e.g., 19,22]. To remove possible contaminants (e.g. amino acids) -resulting in unspecific signals- clean-up columns have been commonly employed prior to HPAEC [e.g., 21,22]. Thus, alternative detection techniques might help to improve the specificity of the analysis.

Liquid chromatography (LC) coupled to mass spectrometry (MS) is a powerful approach for the determination of carbohydrates in freshwater samples [e.g., 6,23]. Methods employing mass spectrometric detection are particularly suitable due to their potential for high sensitivity and specificity of the analyses. In contrast to detection by PAD, molecular identity of the analytes can be confirmed by MS. In addition, different isotopes of a given element can be distinguished, allowing for stable isotope pulse labeling studies to track the distribution and cycling of specific organic compounds in aquatic systems.

The determination of microbial uptake rates of dissolved carbohydrates has been predominantly based on radiolabeled assays [24-27] or cycling parameters of individual compounds have been derived from differences in substrate concentration over time [e.g., 28,29]. Occasionally, stable isotope tracers have been applied to examine the compound dynamics *in situ* [e.g., 30,31,32]. A major advantage of the latter approach is that both, the natural and the added stable isotope-labeled compounds can be monitored simultaneously by MS. Furthermore, stable isotope dilution models [33,34] allow a simultaneous estimation of compound utilization and production rates, which is important for understanding its biogeochemical cycling.

The aim of this study was to develop and validate a rapid and sensitive LC-MS protocol for the determination of dissolved free carbohydrates and amino sugars in diverse freshwater habitats at their low nM concentrations along with a simple isotope dilution approach to quantify the utilization and production rates of selected analytes *in situ*. Here,

we describe the novel method in detail and compare its performance with other approaches for the analysis of the target compounds. Furthermore, we show that our approach allows to simultaneously combine the accurate quantification of carbohydrate and amino sugar concentrations with their cycling rates, thereby providing insight into their *in situ* dynamics.

2. Methods

2.1. Reagents

D-glucose (Glc), D-mannose (Man), D-galactose (Gal), D-fructose (Fru), L-fucose (Fuc), L-rhamnose (Rha), L-arabinose (Ara), D-lyxose (Lyx), D-ribose (Rib), D-xylose (Xyl), cellobiose (Cel), sucrose (Suc), maltose (Mal), N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-mannosamine (ManNAc), N-acetylmuramic acid (MurNAc), N,N`-diacetylglucosamine $(GlcNAc)_2$, sucralose (Scl) were purchased from Sigma-Aldrich at >98% purity grade. LC-MS grade formic acid (50%) and ammonia (25%) were obtained from Sigma-Aldrich. LC-MS grade water (Carl Roth) was used for the preparation of solvents and aqueous reagents. D-[UL-$^{13}C_6$]-glucose (Cambridge Isotope Laboratories) and N-acetyl-D-[UL-$^{13}C_6$]-glucosamine (Omicron Biochemicals) were used to determine the production and utilization rates of Glc and GlcNAc in Lake Zurich.

2.2. Sample collection and preparation

Water samples of 500 ml were collected from the large mesotrophic prealpine Lake Zurich (406 m above sea level, area 65.06 km^2, max. depth 136 m, mean depth 51.7 m, residence time 440 d, 47°17'N, 8°36'E, Switzerland, [35]) into acid-washed glass bottles and were kept at *in situ* temperature in the dark. Additionally, a small eutrophic lake, Hüttensee (658 m above sea level, area 0.165 km^2, max. depth 13.3 m, mean depth 6.3 m, residence time 120 d, 47°14'N, 8°38'E, water quality parameters obtained from the Office of Waste,

Water, Energy and Air, Canton Zurich, http://www.awel.zh.ch/internet/baudirektion /awel/de/wasserwirtschaft/messdaten/see_qualitaet.html) and a small dystrophic peat bog located in the Unterrifferswilermoos area (pH = 4.8, water color – 10.93 mg tannic acid equivalents l^{-1}, 47°14'N, 8°30' E) were sampled once to evaluate the performance of the method on water with a different matrix complexity. Samples were processed by the newly developed LC-MS protocol within 1 h after the sampling. Subsamples of 5 ml were collected using a gas-tight glass syringe (Hamilton) and subsequently filtered through a glass fiber syringe filter (pore size 0.2 µm, diameter 25 mm, Tisch Scientific). Prior to the filtration, the syringe and the filters were first washed once with 2M HCl, then 3 times with sterile deionized water and finally with LC-MS grade water. Filtrates of 1.5 ml were collected into HPLC vials (volume 2 ml, Glastechnik Graefenroda). First 1 ml of the filtrate was discarded. Samples were processed immediately upon filtration or stored at -20°C until further analyses. Visual water color was measured spectrophotometrically at 440 nm and expressed as tannic acid equivalents [36].

2.3. Ion-exclusion chromatography

Carbohydrate analyses were performed with a HPLC system (1260 Infinity series, Agilent Technologies) equipped with a degasser, a binary pump, an autosampler and a column oven. Analytes were separated on a Supelcogel C-610H column (300 x 7.8 mm i.d., 9 µm particle size, sulfonated polystyrene/divinyl benzene, counter ion H^+, Supelco) protected by a column pre-filter (pore size 0.45 µm, Brechbühler). The mobile phase flow rate was 700 µl min^{-1}; it consisted of a gradient of 0.1% formic acid (solvent A) and water (solvent B). The following elution gradient was applied: A from 20 to 50% in 6.9 min, then 50 % A was maintained for 7.4 min, then reduced to 20% A in 0.1 min and this was maintained for 7.6 min. The column temperature was constant at 60°C. Samples were kept at 4°C during

analyses and the injection volume was 50 µl. An internal standard (50 nM sucralose, injection volume 50 µl) was injected from an external vial 4 min after the sample injection by a custom-defined injection sequence. Prior to the first sample injection, the column was equilibrated for 45 min at 20% A until a stable back pressure level was reached, followed by 2 blank injections using the gradient elution as detailed above. To ensure the quality of the separation, retention times of the analytes and the peak area of the internal standard were monitored during analyses.

2.4. Mass spectrometry

Analytes were detected on an API 5000 triple quadrupole mass spectrometer (AB Sciex) equipped with an electrospray ionization probe. Using a multiple reaction monitoring (MRM) scan mode, carbohydrates and amino sugars were detected in the negative and positive ion mode, respectively. Depending on the analyte retention time, only relevant transitions (pairs of precursor and product ions) were monitored at the same time. If required, different MS conditions were applied within a single run to increase the sensitivity of the analysis. The software Analyst (version 1.6.1, AB Sciex) was used for the data acquisition. Measurements of the extracted ion chromatographs (XIC) for each of the monitored transitions were further quantitatively analyzed using the MultiQuant software (version 2.1, AB Sciex). Peak areas (in arbitrary units) were determined by integration algorithms implemented in MultiQuant. The signal to noise ratio threshold was 5.

2.5. Compound optimization assays

Aqueous stock solutions (1 mM) of each compound were stored at -20°C. For the compound optimization assays, 50 to 500 nM working solutions were prepared from the stock solutions. Tandem mass spectrometry (MS/MS) analyses were applied to obtain compound-specific transitions with their specific declustering potential (DP), entering

potential (EP), collision energy (CE) and cell exit potential (CXP) parameters (Table 2). For all transitions EP was set to 10 eV. Firstly, compound working solutions were directly injected into the MS by a syringe pump (Harvard) at a flow rate of 10 µl min^{-1}. Mass spectra (Q1 scan mode) were recorded to confirm the presence of either the deprotonated molecule $[M-H]^-$ in the negative ion mode (neutral carbohydrates) or the protonated molecule $[M+H]^+$ in the positive ion mode (amino sugars). Additionally, product ion mass spectra (product ion scan mode) were acquired from the precursor ion ($[M-H]^-$ or $[M+H]^+$). For all scans, the lower *m/z* threshold was set to 50. To optimize ion-spray voltage (IS), temperature (TEM) as well as curtain (CUR), collision (CAD) and ion source gas (GS1 and GS2) parameters, 10 µl of a compound working solution was injected in 0.05% formic acid as a mobile phase at a flow rate of 700 µl min^{-1}. Signal intensities of the transitions were monitored in the multiple reaction monitoring (MRM) scan mode and compared over a defined range of ion source parameters. Nitrogen was used as curtain, collision and ion source gas. After optimization the ion source parameters for carbohydrates were set as follows: IS = -2500 V, TEM = 600°C, CUR = 35, CAD = 7, GS1 = 57, GS2 = 58. For sucralose (internal standard): IS = -4000 V, TEM = 650°C, CUR = 37, CAD = 8, GS1 = 60, GS2 = 57 and for amino sugars: IS = 4000 V, TEM = 600°C, CUR = 45, CAD = 10, GS1 = 60, GS2 = 55. For each compound, the 2-4 transitions with highest signal intensities were selected. For all transitions the dwell time was set to 100 ms. Transitions will be denoted as in the following example: a molecule with a precursor ion at *m/z* 179 and a product ion at *m/z* 89 will be given as 179/89. Details on the transitions are given in Table 2.

2.6. Internal standard and response factor

For quantitative studies, dilution series of aqueous stock solutions (0.5 – 500 nM) of each analyte and 50 nM solution of sucralose (internal standard) were prepared and

analyzed as described in sections 2.3. and 2.4. Peak areas of both analyte and internal standard were acquired by the MultiQuant software. For each compound, a response factor was calculated as the ratio of the quotient of the analyte and the internal standard peak areas to the quotient of the analyte and the internal standard concentrations. This response factor was then applied to determine the concentration of the target compound in experimental samples.

2.7. Incubation experiments with ^{13}C-labeled Glc and GlcNAc

Water samples from specific depths within the upper 40 m of the water column of Lake Zurich were collected into acid-washed glass bottles (volume, 1 l) in 2013 during the summer stratification and autumn overturn periods. First, the *in situ* concentrations of Glc were assessed in order to yield comparable atom% excess ^{13}C values. Subsequently, triplicate sample bottles were supplemented with D-[UL-$^{13}C_6$]-glucose (final concentrations, 20-30 nM) and N-acetyl-D-[UL-$^{13}C_6$]-glucosamine (final concentrations, 10-20 nM) and compared to duplicated control bottles without substrate amendments. Samples were incubated at *in situ* temperature in the dark for 4 h. Subsamples of 5 ml were taken at intervals of 1 h. Concentrations of natural and ^{13}C-labeled Glc and GlcNAc were determined as described in sections 2.3. and 2.4. Identical pairs of transitions obtained from the natural and ^{13}C-labeled compounds (differences in *m/z* correspond to the number of ^{13}C atoms) were used to determine the atom% excess ^{13}C [30]. The corresponding compound utilization and production rates were calculated according to an isotope dilution model [33,34].

3. Results

3.1. Filtration performance and compound recoveries

Since carbohydrates and amino sugars are readily utilized by microbes and the typical turnover times of these compounds are in the range of hours [e.g., 27] it is essential to stop the biotic reactions by filtration to avoid sample degradation prior to the LC-MS analysis. However, an uncontrolled release of extractable impurities may occur from such filtration devices and interfere with the analysis of targeted analytes [e.g., 37], or analytes can be selectively retained by the filter membranes [e.g., 38]. Therefore, the performance of different membrane types was evaluated: First, ion signal intensities of selected carbohydrate and amino sugar transitions obtained prior and after the filtration of LC-MS grade water were evaluated by the new LC-MS method. Subsequently, the recoveries of selected carbohydrates and amino sugars (0.2-10 nM solutions in LC-MS grade water) were investigated by comparing concentrations before and after the filtration.

Large interferences were observed after filtering the LC-MS grade water through filters of cellulose acetate (CA), nylon, polytetrafluoroethylene (PTFE), and polyvinylidene difluoride (PVDF) membrane types (Table 1). Samples filtered through the glass fiber (GF) and polyethersulfone (PES) filters revealed virtually no or only very minor interferences as compared to the non-filtered LC-MS grade water. Virtually all bacteria and other microbes and particles present in natural samples were removed by 0.22 µm PES filters, whereas only 96-98 % of total bacterial counts could be removed by 0.22 µm GF filters. This was determined by flow cytometry and confirmed by fluorescence microscopy (data not shown). The presence of bacteria in the GF filtrate, however, did not result in significantly different concentrations of the analytes compared to samples filtered through PES filters (data not shown). Nevertheless, PES syringe filters allow for more standardized sample filtration and are, therefore, recommended for sample prefiltration. No significant differences in compound concentrations occurred prior and after the sample filtration through PES

membrane. Recoveries of the analytes ranged from 95 to 104 % at 0.2-10 nM levels (Suppl. Table 1). In addition, the hollow fiber PES filters allow for a very efficient and gentle preparation of large quantities of particle-free water by minimizing the danger of artificial enrichment with solutes due to the breaking of algal cells [39].

3.2. Efficiency of separation and detection

Our chromatographic setup allowed an efficient separation of a number of neutral carbohydrates and amino sugars. The separation factor (α) was determined by comparing the retention factors of two given peaks. Nearly a baseline separation of Glc from other hexoses (α = 1.14) was achieved (Fig. 1A). In contrast, Man, Gal and Fru coeluted (α = 1), and even modified chromatographic conditions did not improve their separation. Fuc and Rha could be well resolved (Fig. 1A, α = 1.16) as well as Xyl, Lyx and Rib (Fig. 1B, α = 1.08 and 1.25, respectively). On the other hand, Ara partly coeluted with Rib (α = 1.03) and Lyx (α = 1.1). Of the tested disaccharides, Cel, Mal and Suc could be resolved although a complete separation was not achieved (Fig. 1B, α = 1.12 and 1.71, respectively). The amino sugars GlcNAc, ManNAc and GalNAc were efficiently separated (Fig. 1C, α = 1.11 and 1.38, respectively). Even though MurNAc and $(GlcNAc)_2$ revealed almost identical retention times (α = 1.06), these compounds could be unambiguously distinguished by their specific transitions (Fig. 1C). Similarly, analytes from different carbohydrate classes (i.e., disaccharides, hexoses, deoxy-hexoses, pentoses) could be accurately determined by MS although the chromatographic conditions applied did not allow for their complete separation. Due to the isomeric nature of the analytes (e.g. hexoses), identical transitions were used for their detection. Unfortunately, the poor chromatographic resolution of Man, Gal and Fru did not allow for their accurate quantification and the compounds could be only partly differentiated based on the relative signal intensities of the transitions.

3.3. Method precision and detection limits

A linear detector response was observed for the studied analytes over a broad range of concentration (0.2-500 nM). Limits of detection (LOD) was determined as the lowest concentration at which the studied analytes yielded a signal to noise ratio of >5 [40]. Depending on the sample types LOD values varied between 0.5-2 nM and between 0.2-1 nM for carbohydrates and amino sugars, respectively. Method precision, assessed as the relative standard deviation (RSD), was calculated from replicate measurements (n = 6) of natural lake water spiked with known concentrations of selected compounds. Limits of quantification (LOQ) were defined as the lowest analyte concentrations with RSD <10 % [41]. The mean RSD values were typically <5 % from 0.2 to 500 nM, as exemplified for Glc and GlcNAc (Fig. 2). For amino sugars, LOD often equaled the LOQ. However, significantly increased RSDs (20-40%) were observed for Glc concentrations below 1-2 nM (Fig. 2). Thus, the effective LOQ values for most identified carbohydrates ranged around 2 nM (Suppl. Table 2).

3.4. Analyses of lake water

Diverse planktonic samples from Lake Zurich were analyzed for the presence of free carbohydrates and amino sugars. From a variety of tested compounds, Glc, Man, Gal, and GlcNAc were readily detected in Lake Zurich. Concentrations of Glc typically ranged from 5 to 30 nM and the summed concentrations of Man, Gal, and Fru usually did not exceed 20 nM. GlcNAc was quantitatively the most important of the tested amino sugars, with a typical concentration range of 0.5-2 nM. Other compounds such as Rha, Ara, Fru, ManNAc, GalNac, $(GlcNAc)_2$, MurNAc and some disaccharides were detected only occasionally or their concentrations were below the detection limit. Even though the distribution and concentrations of Glc and GlcNAc largely varied both spatially and temporally (Fig. 3), the compounds were detected in the vast majority of samples. Therefore, Glc and GlcNAc were

selected as relevant and representative model compounds for carbohydrates and amino sugars, respectively.

3.5. Dynamics of Glc utilization and production in Lake Zurich

To test the applicability of the adopted isotope dilution approach, Glc utilization and production rates were determined in 4 incubation experiments with unfiltered water samples from Lake Zurich during the summer stratification and autumn overturn periods (Table 3). The concentrations of ^{13}C-labeled Glc linearly decreased over time, as illustrated in Fig. 4 for 2 of the experiments, and this decline was steeper in summer samples. In contrast, the concentrations of the unlabeled Glc (i.e., of the natural Glc pool) gradually increased with time, which was paralleled by the increase in Glc concentrations in the controls (Fig. 4). Similar temporal development of the ^{13}C-labeled and natural Glc pools were observed in the other experiments (data not shown). Moreover, significant temporal changes in the ratios of the ^{13}C-labeled and natural Glc resulted in pronounced changes in the total Glc pool size and atom% excess ^{13}C values implying differences in Glc utilization and production rates (Fig. 4). The latter also pointed at rapid recycling of Glc. While Glc utilization or production rates did not correlate with depth, temperature nor with chlorophyll *a* values, both rates were significantly higher during summer stratification than in the autumn overturn period (paired t-test, $P < 0.01$, $n = 6$).

3.6. Samples from eutrophic and dystrophic habitats

To evaluate the performance of the new method on environmental samples with a different matrix complexity as compared to the mesotrophic Lake Zurich, water samples were collected from the eutrophic Hüttensee and the dystrophic peat bog Unterrifferswilermoos (Fig. 5). The carbohydrate composition in these habitats was similar to that of Lake Zurich: While Glc, Man, Gal, Fru were also detected in eutrophic Hüttensee and

298 in the peat bog, an unidentified disaccharide was present in both habitats. Concentrations of
299 amino sugars were below their detection limits during the time of sampling, apart from 5 nM
300 GlcNAc detected in the peat bog. In general, highest concentrations of the targeted analytes
301 were found in the dystrophic peat bog (up to 50 nM Glc as well as of a mix of Man, Gal, Fru)
302 followed by eutrophic and mesotrophic habitats. Most importantly, no significant
303 interferences in carbohydrate and amino sugar signal intensities as well as in background
304 noise levels were observed in any of the tested samples (Fig. 5). This suggests that the
305 detection limits of the targeted analytes by the here presented method are generally low in
306 freshwater habitats with different trophic states and matrix complexities.
307
308 **4. Discussion**
309 *4.1. Method optimization*
310 Our goal was to develop a method to simultaneously detect dissolved free
311 carbohydrates and amino sugars in freshwater samples at their expected *in situ*
312 concentrations that would be both, sufficiently sensitive and without time-consuming
313 sample preparation. We also aimed at avoiding any sample cleanup steps that could
314 compromise the quality of analyses and recovery of the analytes. Due to the high specificity
315 and the possibility of analyzing different isotopes, mass spectrometry (MS) was selected as a
316 superior detection system to the conventionally used pulse amperometric detectors [18].
317 Since our investigation focused on low-molecular weight compounds, the system of choice
318 was a triple quadrupole mass spectrometer with excellent resolution of the targeted
319 analytes. In contrast, Fourier-transform mass spectrometry, which has become increasingly
320 popular, is particularly promising for analyses of large and complex molecules [e.g., 42].

Application of MS was also the reason why we did not choose the well-established separation of carbohydrates and amino sugars by anion-exchange chromatography under basic conditions [e.g., 18,21]. The HPAEC method has been previously combined with MS [e.g., 6,22,43,44] or with isotope ratio mass spectrometry [45] to determine carbohydrates in a variety of samples such as bacterial cell hydrolysates, food and beverage products, plant tissue or marine sediments. However, the above studies all required a desalting step or additional removal of contaminants (e.g., amino acids) prior to the detection. To our knowledge, only one study [6] has applied the HPAEC-MS method for the determination of free carbohydrates and other low-molecular-weight compounds in freshwater samples, albeit without providing sufficient details for easy reproduction: Thus, despite its apparent potential, there were considerable obstacles to adopt the method by Berggren and coworkers [6] for our routine analyses of free carbohydrates without extensive testing. Therefore, in order to find a suitable chromatographic setup readily compatible with MS, other approaches were explored, such as normal-phase chromatography (NPC) and ion-exclusion chromatography (IEC).

While targeted analytes with different molecular weights could be well resolved by NPC using a silica resin coated with an amino phase, separation of isobaric compounds was not satisfactory even when modified gradient elutions with volatile buffers at different concentrations were used (data not shown). In contrast, carbohydrates (Fig. 1A,B) and amino sugars (Fig. 1C) could be effectively separated on a strong cation exchange resin by IEC. Moreover, the applied chromatographic conditions proved to be very stable, and no significant shifts in analyte retention times was observed over a 1 year period of intense column use. The new protocol also allowed for higher throughput, i.e., of 3 samples per hour, which is significantly faster than previously reported [9]. IEC coupled to MS permitted

a rapid and sensitive detection of carbohydrates and amino sugars at low nM concentrations (Fig. 2). For some analytes, such as amino sugars, even sub-nM concentrations could be quantified in lake water (Fig. 3B). Detection and quantification limits are, thus, comparable to the threshold concentrations reported for the HPAEC-PAD method [18].

It should be noted that the HPAEC method may indeed allow for a better separation than IEC. However, the co-eluting compounds from different classes (e.g., hexose vs deoxy-hexose) could still be unambiguously discriminated by their specific transitions in our approach. Thus, the incomplete chromatographic separation could be effectively compensated for by MS detection. The difficulties related to the poor chromatographic resolution of Man, Gal and Fru (i.e., compounds within the same class) might be circumvented by using other available columns that show a baseline separation of these analytes, such as the Supelcogel Pb column (Supelco).

4.2. Stable isotope tracers

Despite the high detection potential of our approach, it was not our intention to develop a screening method for the presence of a large range of sugars in freshwaters. Instead, chromatographic conditions were specifically adjusted toward a baseline separation of selected compounds such as Glc and GlcNAc to be studied in more detail. In particular, we aimed at combining the accurate concentration measurement of specific carbohydrates and amino sugars with their *in situ* dynamics [e.g., 46,47]. We, therefore, adopted the isotope dilution technique [30,33,34] to track the utilization and production rates of Glc and GlcNAc in Lake Zurich at low nM concentration ranges.

From our incubation experiments with stable isotope tracers (Fig. 4), utilization and production rates of Glc could be simultaneously determined (Table 3), thus providing valuable information on the *in situ* cycling of this compound. The isotope dilution model

provides a reliable estimate of the cycling rates despite a negligible difference in the total compound pool during the incubation period [48]. This allows for the estimation of rates even at low nM concentration range. To date, knowledge about the *in situ* dynamics of particular substrate in freshwaters largely originates from determining its concentration and uptake rate only [e.g., 8,20,49]. However, our incubation experiments suggest that the *in situ* pool of dissolved free Glc in Lake Zurich is not at steady state conditions. This emphasizes the importance of measuring both, uptake and production rates to understand the cycling of a particular compound. Furthermore, the model uses the isotope atom% excess values in order to determine whether and to which extent the compound was turned over. This is particularly important since the uptake of a given compound does not necessarily imply its recycling. For instance, performing parallel experiments with ^{13}C-labeled GlcNAc revealed a rapid utilization of this compound, whereas no measurable production of GlcNAc could be detected (Suppl. Fig. 1). Thus, despite comparable utilization rates, Glc and GlcNAc revealed distinctly different *in situ* dynamics.

The concentrations of dissolved free GlcNAc likely corresponded to ~1% of the expected total amino sugar pool size in Lake Zurich, as deduced from the amino sugar concentrations in the total organic matter pools of two other Swiss lakes [50]. However, this is not surprising since amino sugars are principal components of structural biopolymers such as peptidoglycans or chitin that are typically found in the particulate fraction of organic matter, for instance, in bacterial, cyanobacterial, and algal cell walls or in zooplankton exoskeleton [51-54]. Since the focus of our study was exclusively on dissolved compounds that would be readily available to free-living microbial cells, these organic particles were removed by filtration prior to analyses. Overall, the use of stable isotope tracers together with accurate measurements of concentrations by MS allows for a highly specific and

sensitive analysis of the *in situ* dynamics and microbial uptake of various dissolved compounds and provides a convenient alternative to the radiolabeled incubation assays.

4.3. Pitfalls

Incubation studies with ^{13}C-labeled tracers at close to *in situ* (nM) concentrations are delicate, and it is recommended to tightly control the sample handling, filtration and storage to prevent sample contamination or degradation. The samples should not be exposed to large temperature changes, which can significantly affect microbial processes such as substrate utilization rates or carbon metabolism [e.g., 55,56]. The incubations should be started as soon as possible (preferably within 1 h) after sampling, because the composition and activity of the microbial assemblage may rapidly change after being disconnected from the natural environment [e.g., 57,58]. Finally, since the utilization and production rates are calculated from the change in concentrations of natural and labeled compounds over time, a linear relationship between the latter parameters is required. Thus, in order to properly determine atom% excess of ^{13}C, incubation time and sampling frequency should be adjusted accordingly.

4.4. Conclusions

The here described new procedure is characterized by rapid sample preparation and highly accurate quantification by MS. Using direct sample injections, a range of dissolved free carbohydrates and amino sugars can be detected and separated at low nM concentrations in a variety of freshwater samples including eutrophic and dystrophic habitats (Fig. 5). Short-termed incubation experiments with stable isotope tracers provide a suitable tool for studying the microbial substrate processing.

Acknowledgments

We thank E. Loher and M. Steinkellner for their help with field sampling and M. Scherer (AB Sciex, Switzerland) for the introduction into mass spectrometry. We also thank three anonymous reviewers for their constructive criticisms of the original submission. Funding for this work was provided by the Swiss National Science Foundation under the project 31003A-141166.

References

[1] B. Riemann, M. Søndergaard, L. Persson, L. Johansson, Carbon metabolism and community regulation in eutrophic, temperate lakes, in: B. Riemann, M. Søndergaard (Eds.), Carbon dynamics in eutrophic, temperate lakes, Elsevier, Amsterdam, 1986, p. 267-280.

[2] F. Azam, T. Fenchel, J.G. Field, J.S. Gray, L.A. Meyer-Reil, F. Thingstad, The ecological role of water-column microbes in the sea, Mar. Ecol. Prog. Ser. 10 (1983) 257-263.

[3] L.J. Tranvik, M.G. Höfle, Bacterial growth on mixed cultures on dissolved organic carbon from humic and clear waters, Appl. Environ. Microbiol. 53 (1987) 482-488.

[4] S.R. Carpenter, J.J. Cole, M.L. Pace, M. Van De Bogert, D.L. Bade, D. Bastviken, C.M. Gille, J.R. Hodgson, J.F. Kitchell, E.S. Kritzberg, Ecosystem subsidies: terrestrial support of aquatic food webs from C-13 addition to contrasting lakes, Ecology 86 (2005) 2737-2750.

[5] J.J. Cole, S.R. Carpenter, M.L. Pace, M.C. Van de Bogert, J.L. Kitchell, J.R. Hodgson, Differential support of lake food webs by three types of terrestrial organic carbon, Ecology Letters 9 (2006) 558-568.

[6] M. Berggren, H. Laudon, M. Haei, L. Ström, M. Jansson, Efficient aquatic bacterial metabolism of dissolved low-molecular-weight compounds from terrestrial sources, ISME J. 4 (2010) 408-416.

[7] R. Benner, K. Kaiser, Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter, Limnol. Oceanogr. 48 (2003) 118-128.

[8] L.J. Tranvik, N.O.G. Jørgensen, Colloidal and dissolved organic matter in lake water: Carbohydrate and amino acid composition, and ability to support bacterial growth., Biogeochemistry 30 (1995) 77-97.

[9] C. Panagiotopoulos, R. Sempéré, Analytical methods for the determination of sugars in marine samples: A historical perspective and future directions, Limnol. Oceanogr-Meth. 3 (2005) 419-454.

[10] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugar and related substances, Analytical Chemistry 28 (1956) 350-356.

[11] S.V. Myklestad, E. Skånøy, S. Hestmann, A sensitive method for analysis of dissolved mono- and polysaccharides in seawater, Analytical Chemistry 56 (1997) 279-286.

[12] E. Sawicki, T.R. Hauser, T.W. Stanley, W. Elbert, The methyl-2-benzothiazolone hydrazone test, Anal. Chem. 33 (1961) 93-96.

[13] K. Mopper, Improved chromatographic separation on anion-exchange resins. III. Sugars in borate medium, Anal. Biochem. 87 (1978) 162-168.

[14] K. Mopper, R. Dawson, G. Liebezeit, H.P. Hansen, Borate complex ion exchange chromatography with fluorimetric detection for determination of saccharides, Anal. Chem. 52 (1980) 2018-2022.

[15] A. Meyer, C. Raba, K. Fischer, Ion-pair RP-HPLC determination of sugars, amino sugars and uronic acids after derivatization with p-amino benzoic acid, Anal. Chem. 73 (2001) 2377-2382.

[16] G.L. Cowie, J.I. Hedges, Determination of neutral sugars in plankton, sediments, and wood by capillary gas chromatography of equilibrated isomeric mixtures, Anal. Chem. 56 (1984) 497-504.

[17] H. Sakugawa, N. Handa, Chemical studies on dissolved carbohydrates in water samples collected from North Pacific and Bering Sea., Oceanol. Acta 8 (1985) 185-196.

[18] K. Mopper, C.A. Schultz, L. Chevolot, C. Germain, R. Revueta, R. Dawson, Determination of sugars in unconcentrated seawater ad other natural waters by liquid chromatography and pulsed amperometric detection, Environ. Sci. Technol. 26 (1992) 133-138.

[19] K. Kaiser, R. Benner, Determination of amino sugars in environmental samples with high salt content by high-performance anion-exchange chromatography and pulsed amperometric detection Anal. Chem. 72 (2000) 2566-2572.

[20] N.O.G. Jørgensen, R.E. Jensen, Microbial fluxes of free monosaccharides and total carbohydrates in freshwater determined by PAD-HPLC, FEMS Microb. Ecol. 14 (1994) 79-94.

[21] X. Cheng, L.A. Kaplan, Simultaneous analyses of neutral carbohydrates and amino sugars in freshwaters with HPLC-PAD, J. Chromatogr. Sci. 41 (2003) 1-5.

[22] D.S. Wunschel, K.F. Fox, A. Fox, M.L. Nagpal, K. Kim, G.C. Stewart, M. Shahgholi, Quantitative analysis of neutral and acidic sugars in whole cell hydrolysates using high-performance anion-exchange liquid chromatography-electrospray ionization tandem mass spectrometry, J. Chromatogr. A 776 (1997) 205-219.

[23] B. Meon, F. Jüttner, Concentrations and dynamics of free mono- and oligosaccharides in a shallow eutrophic lake measured by thermospray mass spectrometry, Aquat. Microb. Ecol. 16 (1999) 281-293.

[24] C. Alonso, M. Zeder, C. Piccini, D. Conde, J. Pernthaler, Ecophysiological differences of betaproteobacterial populations in two hydrochemically distinct compartments of a subtropical lagoon, Environ. Microbiol. 11 (2009) 867-876.

[25] U. Buck, H.-P. Grossart, R. Amann, J. Pernthaler, Substrate incorporation patterns of bacterioplankton populations in stratified and mixed waters of a humic lake, Environ. Microbiol. 11 (2009) 1854-1865.

[26] R.R. Wright, J.E. Hobbie, Use of glucose and acetate by bacteria and algae in aquatic ecosystems, Ecology 47 (1966) 447-464.

[27] C. Bunte, M. Simon, Bacterioplankton turnover of dissolved free monosaccharides in a mesotrophic lake, Limnol. Oceanogr. 44 (1999) 1862-1870.

[28] R.M.W. Amon, R. Benner, Rapid cycling of high-molecular-weight dissolved organic matter in the ocean, Nature 369 (1994) 549-552.

[29] D.L. Kirchman, Y. Suzuki, C. Garside, H.W. Ducklow, High turnover rates of dissolved organic carbon during a spring phytoplankton bloom, Nature 352 (2001) 612-614.

[30] O. Köster, F. Jüttner, NH_4^+ utilization and regeneration rates in freshwater lakes determined by GC-MS of derivatized dihydroindophenol, J. Microbiol. Meth. 37 (1999) 65-76.

[31] T. Hama, K. Yanagi, Production and neutral aldose composition of dissolved carbohydrates excreted by natural marine phytoplankton populations, Limnol. Oceanogr. 46 (2001) 1945-1955.

[32] T. Hasegawa, H. Fukuda, I. Koike, Effects of glutamate and glucose on N cycling and the marine plankton community, Aquat. Microb. Ecol. 41 (2005) 125-130.

[33] T.H. Blackburn, Method for measuring rates of NH_4^+ turnover in anoxic marine sediments, using a ^{15}N-NH_4^+ dilution technique, Appl. Environ. Microb. 37 (1979) 760-765.

[34] J. Caperon, D. Schell, J. Hirota, E. Laws, Ammonim excretion rates in Kaneohe Bay, Hawaii, measured by a ^{15}N isotope dilution technique, Mar. Biol. 54 (1979) 33-40.

[35] P. Bossard, S. Gammeter, C. Lehmann, F. Schanz, R. Bachofen, H.-R. Bürgi, D. Steiner, U. Zimmermann, Limnological description of the Lakes Zürich, Lucerne, and Cadagno, Aquat. Sci. 63 (2001) 225-249.

[36] I.D. Cuthbert, P. del Giorgio, Toward a standard method of measuring color in freshwater, Limnol. Oceanogr. 37 (1992) 1319-1326.

[37] J.C. Tran, A.A. Doucette, Cyclic polyamide oligomers extracted from nylon 66 membrane filter disks as a source of contamination in liquid chromatography/mass spectrometry, J. Am. Soc. Mass Spectr. 17 (2006) 652-656.

[38] M. Lindenberg, C. Wiegand, J.B. Dressman, Comparison of the adsorption of several drugs to typical filter materials, Dissolut. Technol. 12 (2005) 22-25.

[39] F. Jüttner, B. Meon, O. Köster, Quasi *in situ* separation of particulate matter from lakewater by hollow-fibre filters to overcome errors caused by short turnover times of dissolved compounds, Water Res. 31 (1997) 1637-1642.

[40] A. Shrivastava, V.B. Gupta, Methods for the determination of limit of detection and limit of quantification of the analytical methods, Chron. Young Sci. 2 (2011) 21-25.

[41] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantification, Clin. Biochem. Rev. 29(Suppl 1) (2008) S49-S52.

[42] K.D. Henry, E.R. Williams, B.H. Wang, F.W. McLafferty, J. Shabanowitz, D.F. Hunt, Fourier-transform mass spectrometry of large molecules by electrospray ioization, P. Natl. Acad. Sci. USA 86 (1989) 9075-9078.

[43] C. Bruggink, R. Maurer, H. Herrmann, S. Cavalli, F. Hoefler, Analysis of carbohydrates by anion exchange chromatography and mass spectrometry, J. Chromatogr. A 1085 (2005) 104-109.

[44] T. Kirchgeorg, S. Schüpbach, N. Kehrwald, D.B. McWethy, C. Barbante, Method for the determination of specific molecular markers of biomass burning in lake sediments, Org. Geochem. 71 (2014) 1-6.

[45] H.T.S. Boschker, T.C.W. Moerdijk-Poortvliet, P. van Breugel, M. Houtekamer, J.J. Middelburg, A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Sp. 22 (2008) 3902-3908.

[46] J. Nedoma, J. Vrba, J. Hejzlar, K. Šimek, V. Straškrabová, N-acetylglucosamine dynamics in freshwater environments: Concentration of amino sugars, extracellular enzyme activities, and microbial uptake, Limnol. Oceanogr. 39 (1994) 1088-1100.

[47] H.-P. Grossart, A. Engel, C. Arnosti, C.L. De La Rocha, A.E. Murray, U. Passow, Microbial dynamics in autotrophic and heterotrophic seawater mesocosms. III. Organic matter fluxes, Aquat. Microb. Ecol. 49 (2007) 143-156.

[48] E. Laws, Isotope dilution models and the mystery of vanishing ^{15}N, Limnol. Oceanogr. 29 (1984) 379-386.

[49] M. Weiss, M. Simon, Consumption of labile dissolved organic matter by limnetic bacterioplankton: the relative significance of amino acids and carbohydrates, Aquat. Microb. Ecol. 17 (1999) 1-12.

[50] D. Carstens, K.E. Köllner, H. Bürgmann, B. Wehrli, C.J. Schubert, Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids, Geochim. Cosmochim. Acta 89 (2012) 159-172.

[51] J. Blackwell, K.D. Parker, K.M. Rudall, Chitin fibers of the diatom *Thalassiosira fluviatilis* and *Cyclotella cryptica*, J. Molec. Biol. 28 (1967) 383-385.

[52] I.W. Sutherland, Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides, Annu. Rev. microbiol. 39 (1985) 243-270.

[53] E. Cohen, Chitin biochemistry: synthesis and inhibition, Annu. Rev. Entomol. 32 (1987) 71-93.

[54] C. Bertocchi, L. Navarini, A. Cesàro, Polysaccharides from cyanobacteria, Carbohydr. Polym. 12 (1990) 127-153.

559 [55] J.K. Apple, P. Del Giorgio, W.M. Kemp, Temperature regulation of bacterial production,
560 respiration, and growth efficiency in a temperate salt-marsh estuary, Aquat. Microb. Ecol. 43
561 (2006) 243-254.
562 [56] C.C. Crawford, J.E. Hobbie, K.L. Webb, The utilization of dissolved free amino acids by
563 estuarine microorganisms, Ecology 55 (1974) 551-563.
564 [57] E.B. Sherr, B.F. Sherr, C.T. Sigmon, Activity of marine bacteria under incubated and *in situ*
565 conditions, Aquat. Microb. Ecol. 20 (1999) 213-223.
566 [58] R. Massana, C. Pedrós-Alió, E.O. Casamayor, J.M. Gasol, Changes in marine bacterioplankton
567 phylogenetic composition during incubations designed to measure biogeochemically
568 significant parameters, Limnol. Oceanogr. 46 (2001) 1181-1188.

569 **Figures captions**

570 Fig. 1. LC-MS extracted ion chromatograms of a mixture (100 nM each) of hexoses and
571 deoxy-hexoses (A), disaccharides and pentoses (B), and amino sugars (C) acquired using a
572 multiple reaction monitoring (MRM) scan mode. Identical transitions were applied for
573 isobaric analytes. Glc - glucose, Man - mannose, Gal - galactose, Fru - fructose, Fuc - fucose,
574 Rha - rhamnose, Scl – sucralose, Cel - cellobiose, Mal - maltose, Suc - sucrose, Xyl - xylose,
575 Lyx - lyxose, Ara - arabinose, Rib - ribose, MurNAc - N-acetylmuramic acid, $(GlcNAc)_2$ - N,N`-
576 diacetylglucosamine, ManNAc - N-acetyl-D-mannosamine, GlcNAc - N-acetyl-D-glucosamine,
577 GalNAc - N-acetyl-D-galactosamine.

578

579 Fig. 2. Calibration curves of Glc (A) and GlcNAc (B) to determine the limits of detection (LOD)
580 and limits of quantification (LOQ) in samples from Lake Zurich. Values are means of 6

581 repetitive scans. Error bars show relative standard deviation. Arrows indicate the
582 corresponding LOD and LOQ values.
583
584 Fig. 3. Concentrations of dissolved free Glc and GlcNAc in selected samples from Lake Zurich
585 on 18 April (A) and 08 May (B) 2013. Values are means of triplicates. Error bars show
586 standard deviations. Note different scales of Glc concentrations.

587 Fig. 4. Temporal changes in the concentrations of total, natural (^{12}C) and ^{13}C-labeled Glc in
588 selected experimental incubations performed with epilimnetic samples (6 and 12 m) from
589 Lake Zurich taken during summer stratification (A) and autumn overturn (B) periods in 2013.
590 A linear fit of the total Glc concentrations (as a sum of natural and ^{13}C-labeled Glc pools) is
591 shown. Bars represent the concentrations of Glc in the control incubations that were not
592 amended with the ^{13}C-labeled Glc. Values are means of triplicates. Error bars show standard
593 deviations (range is shown for controls). The corresponding plots of ln(atom% excess ^{13}C)
594 against $\ln(Glc_t / Glc_{t0})$ from the same incubations during summer stratification (C) and
595 autumn overturn (D). Glc_t – concentration of total Glc at different time intervals, Glc_{t0} –
596 initial total Glc concentration.
597

598 Fig. 5. LC-MS total ion chromatograms (TIC) of selected carbohydrates and amino sugars
599 detected in the mesotrophic Lake Zurich (A), eutrophic Hüttensee (B), and dystrophic
600 Unterrifferswilermoos peat bog (C) in March 2014. Peak identification: (1) unknown
601 disaccharide, (2) glucose, (3) galactose, mannose, fructose, (4) N-acetyl-glucosamine, (5)
602 sucralose (internal standard). Different mass spectrometric settings were applied to detect
603 carbohydrates, N-acetyl-amino sugars and internal standard between 0-10, 10-12, and 12-15
604 min, respectively. Note different scales of ion signal intensities.
605

Highlights:

- Novel HPLC-MS method for carbohydrate and amino sugar analysis in freshwaters
- Rapid and efficient determination without derivatization or sample pre-concentration
- Implementation of stable isotope tracers to study compound biogeochemical cycling
- Glucose and N-acetyl-glucosamine showed different in situ dynamics in Lake Zurich

FIG1

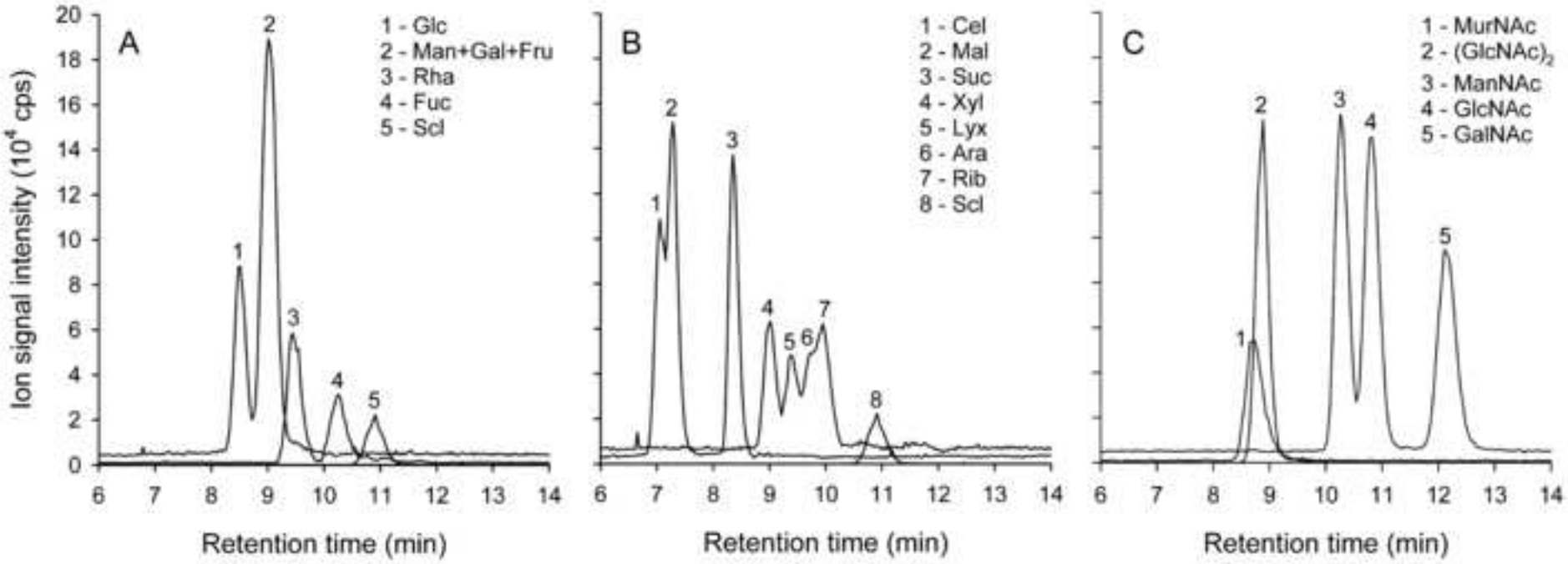


FIG2

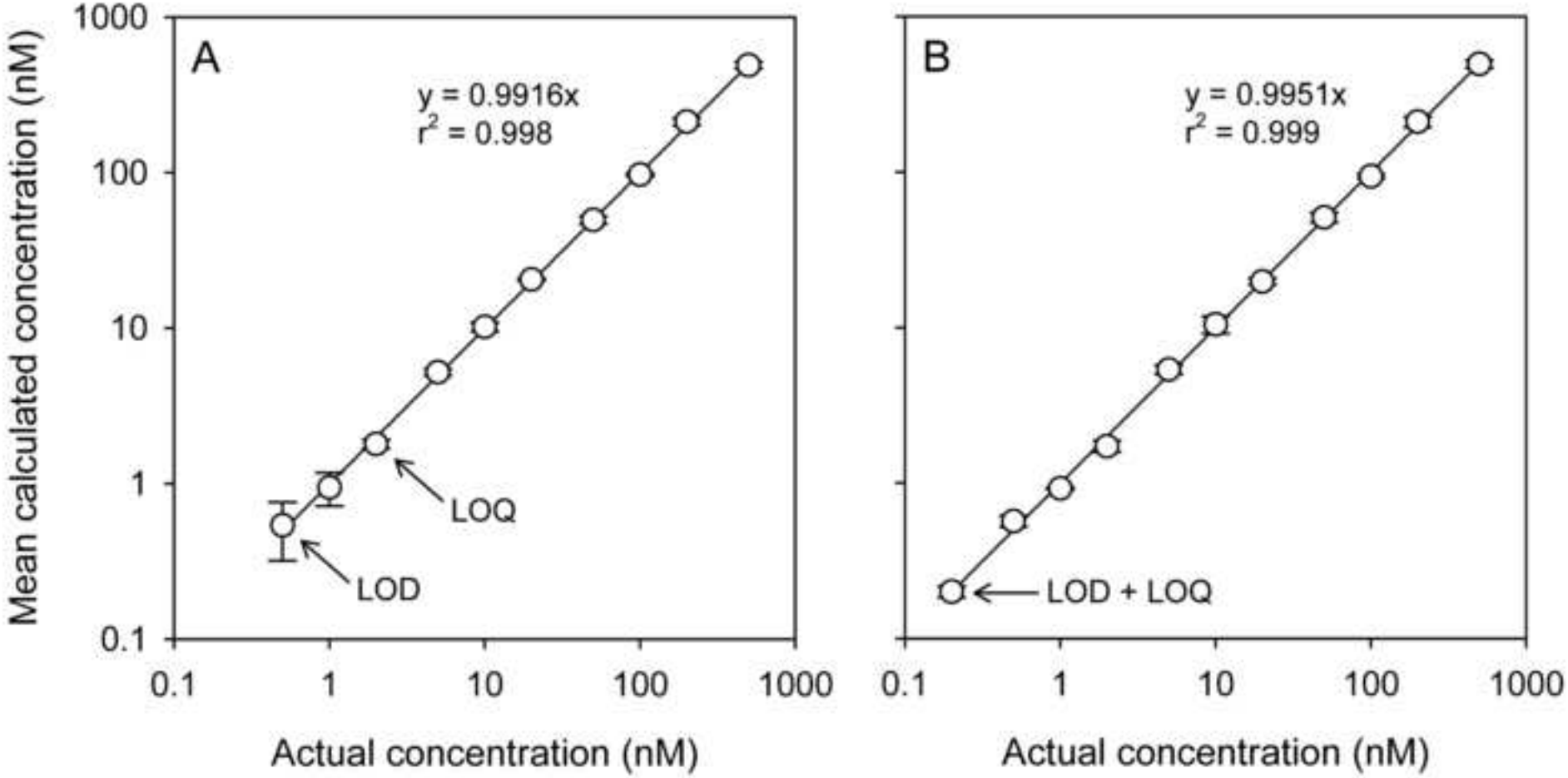
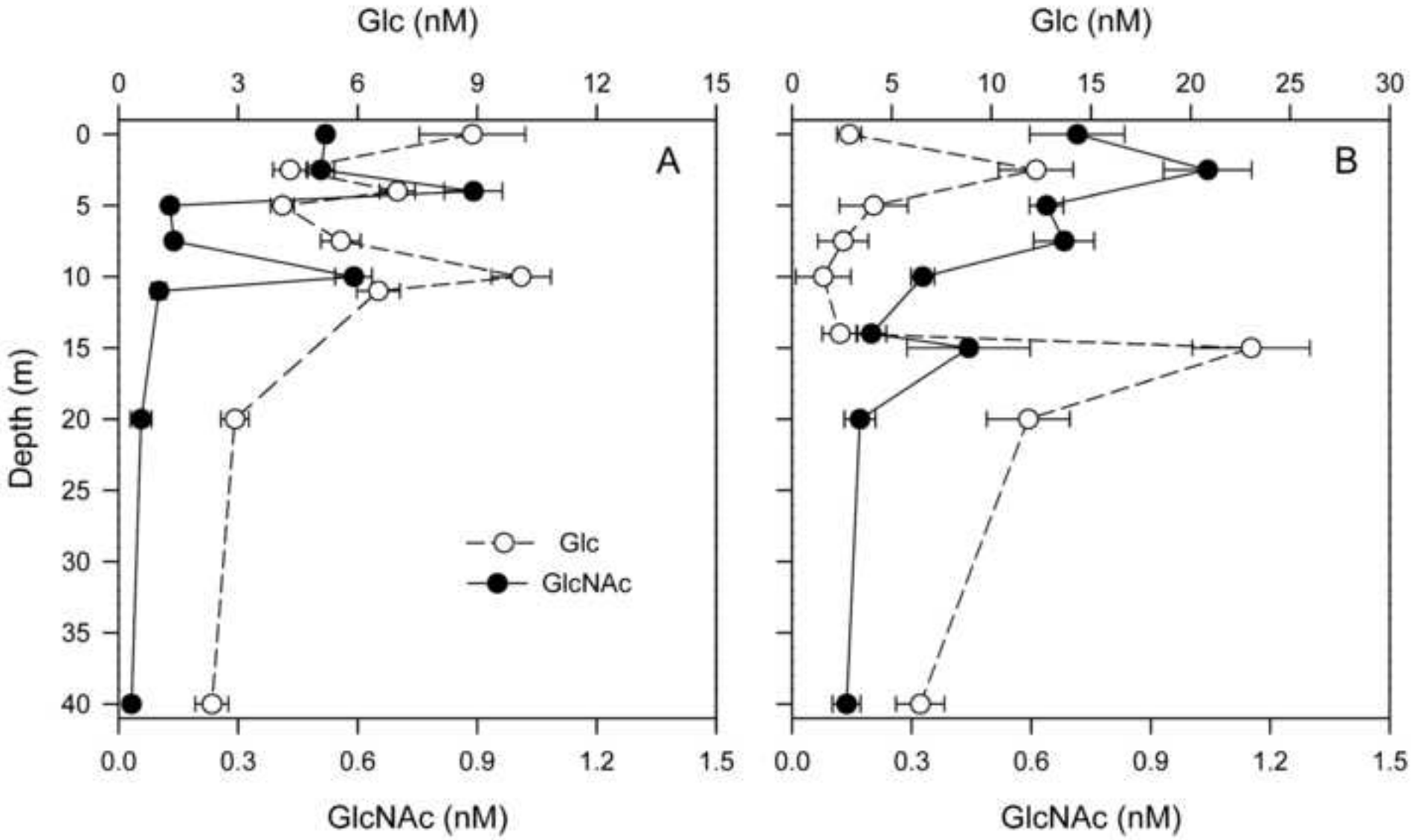

A
y = 0.9916x
r² = 0.998
LOQ
LOD
Mean calculated concentration (nM)
Actual concentration (nM)
1000
100
10
1
0.1
0.1
1
10
100
1000
B
y = 0.9951x
r² = 0.999
LOD + LOQ
Actual concentration (nM)

FIG3


Glc (nM)
0
3
6
9
12
15
A
Depth (m)
0
5
10
15
20
25
30
35
40
Glc
GlcNAc
0.0
0.3
0.6
0.9
1.2
1.5
GlcNAc (nM)
Glc (nM)
0
5
10
15
20
25
30
B
0.0
0.3
0.6
0.9
1.2
1.5
GlcNAc (nM)

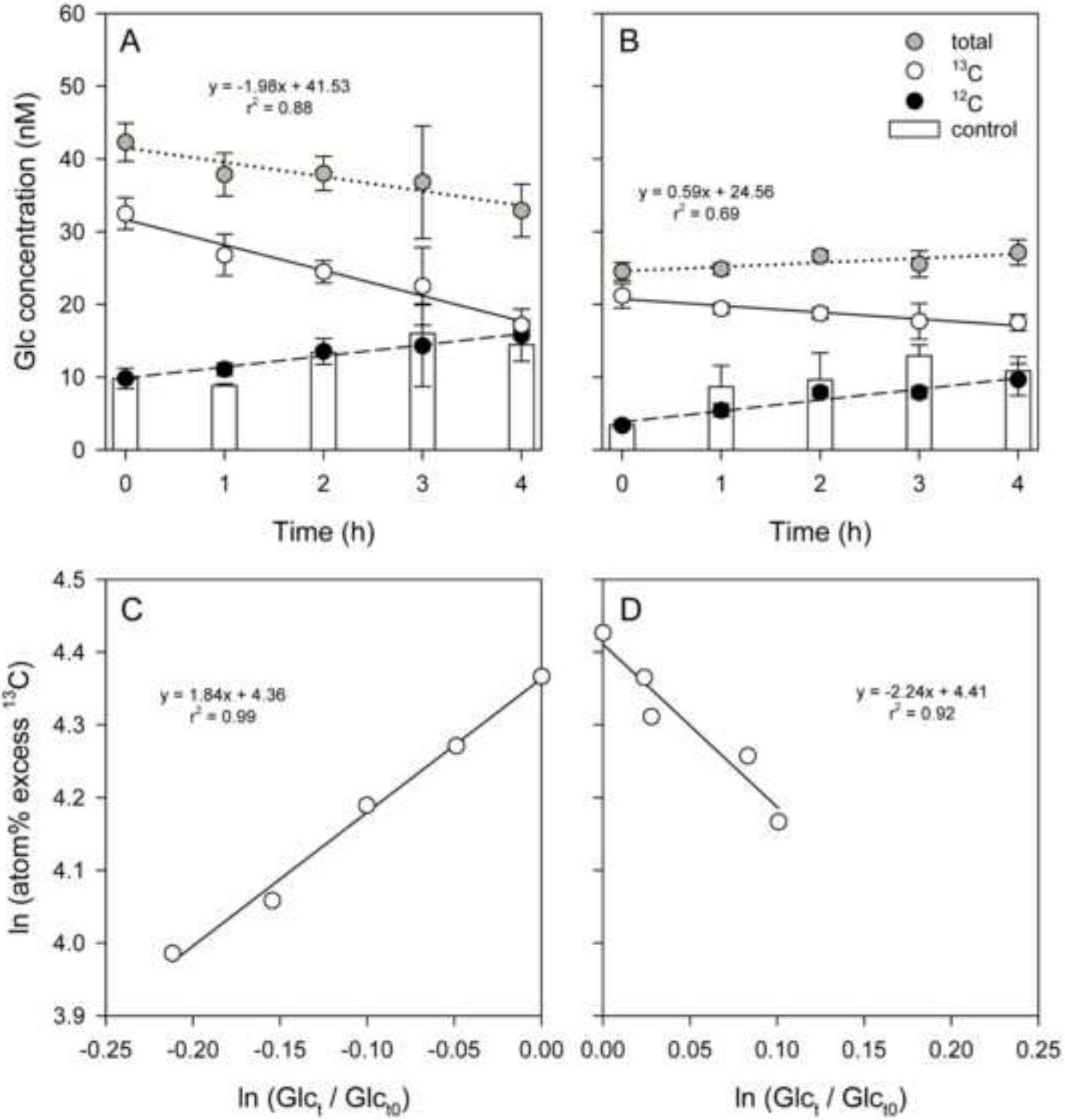
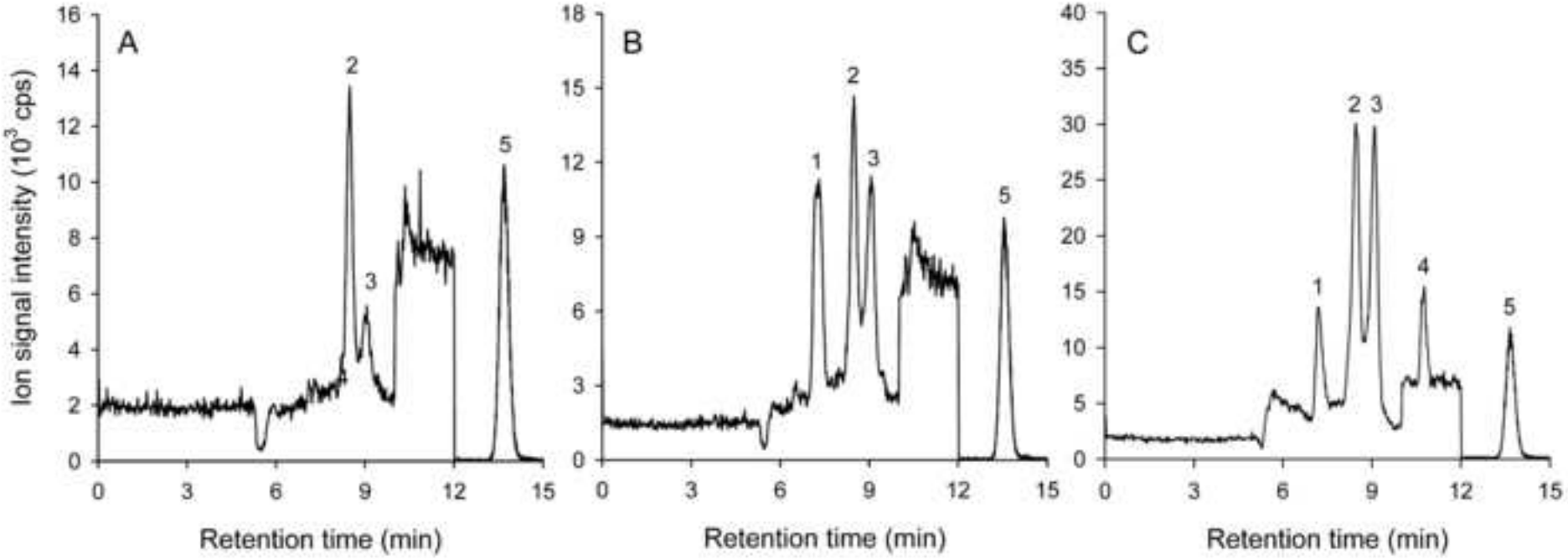


FIG5



Tables

Table 1: Evaluation of syringe filters with different membrane types used for sample filtration prior to HPLC-MS analyses of carbohydrates and amino sugars. CA – cellulose acetate, GF – glass fiber, NY – nylon, PES – polyethersulphone, PVDF – polyvinylidene fluoride, PTFE – polytetrafluoroethylene. Recommended filters are highlighted in bold.

Membrane type	Pore size & diameter	Manufacturer
CA	0.22 µm, 25 mm	Whatman
GF	0.22 µm, 25 mm	Tisch Scientific
NY	0.22 µm, 25 mm	Millipore
PES	**0.22 µm, 13 mm**	**Pall**
PES (hollow fiber)	**0.05 & 0.22 µm**	**Spectrum**
PVDF	0.1 µm, 25 mm	Millipore
PTFE	0.22 µm, 25 mm	Whatman

Table 2: Multiple reaction monitoring (MRM) parameters applied to detect carbohydrates and internal standard (Scl, negative ion mode) and amino sugars (positive ion mode). Transitions (pairs of precursor and product ions) and the corresponding declustering potential (DP), collision energy (CE) and cell exit potential (CXP) parameters are shown. Gal - galactose, Glc - glucose, Fru - fructose, Man - mannose, Fuc - fucose, Rha - rhamnose, Ara - arabinose, Lyx - lyxose, Rib - ribose, Xyl - xylose, Cel - cellobiose, Mal - maltose, Suc - sucrose, Scl – sucralose, GalNAc - N-acetyl-D-galactosamine, GlcNAc - N-acetyl-D-glucosamine, ManNAc - N-acetyl-D-mannosamine, $(GlcNAc)_2$ - N,N`-diacetylglucosamine, MurNAc - N-acetylmuramic acid.

Compound	DP (V)	Transitions (m/z)	CE (V)	CXP (V)
Gal, Glc, Fru, Man	-75	179/59	-22	-5
		179/71	-22	-9
		179/89	-14	-13
		179/119	-8	-19
Fuc, Rha	-55	163/59	-22	-5
		163/101	-12	-15
Ara, Lyx, Rib, Xyl	-75	149/59	-18	-7
		149/71	-12	-13
		149/89	-10	-7
Cel, Mal, Suc	-75	341/59	-44	-9
		341/101	-22	-9
		341/161	-8	-11
		341/179	-8	-21
Scl (internal standard)	-150	397/361	-14	-15
		397/359	-16	-19
		395/359	-10	-19
GalNAc, GlcNAc, ManNAc	36	222/126	17	14
		222/138	21	20
		222/144	21	10
		222/186	15	12
$(GlcNAc)_2$	126	425/84	59	18
		425/126	35	18
		425/204	15	22
MurNAc	66	294/84	29	16
		294/114	41	14

Table 3: Dynamics of Glc in selected samples from Lake Zurich during summer stratification (August) and autumn overturn (October) periods in 2013. The corresponding *in situ* temperature, chlorophyll *a* and Glc concentrations are shown. Glc utilization and production rates were obtained from the incubation experiments with ^{13}C-labeled Glc.

Sampling date	Depth (m)	Temperature (°C)	Chl *a* (µg l^{-1})	Glc (nM)	Utilization rate (nM Glc h^{-1})	Production rate (nM Glc h^{-1})
05 Aug 2013	6	25	5	9.8	3.65	1.66
	12	14	34	8.6	5.65	3.09
	20	7	4	3.5	3.33	2.94
12 Aug 2013	6	24	6	5.5	5.89	6.22
	12	13	33	9	5.14	2.76
	22	7	3	9.4	1.75	2.63
21 Oct 2013	5	15	17	3.3	0.87	1.57
	10	15	17	5.7	0.53	0.91
	40	5	0.4	3.9	1.68	1.65
28 Oct 2013	5	14	19	2.8	2.68	1.51
	10	14	17	1.5	1.96	1.24
	40	5	0.6	7	1.74	0.61